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FOREWORD

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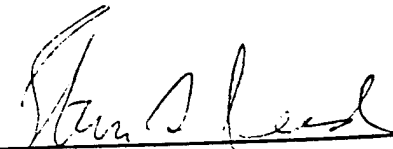
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(5) INTRODUCTION

Breast cancer is a disease where breast epithelial cells become refractory to appropriate growth and differentiation signals. It is likely that numerous genetic changes can contribute to malignant transformation, including mutations that alter the cell cycle regulatory machinery. We have therefore sought to characterize the function of both positive and negative cell cycle regulatory elements in normal and malignant breast epithelial cells. In particular, we have focused on cyclin E, a positive cell cycle regulatory element already implicated in some breast malignancies and a class of negative regulators of cyclin dependent kinases (Cdks). To study the role of cyclin E in breast malignancy, we screened cell lines derived from breast carcinomas for cyclin E mutations. Additionally we have targeted cyclin E using antisense strategies to examine its essentiality. To investigate the role of Cdk inhibitors in regulation of proliferation of breast epithelial cells, we have (1) identified Cdk inhibitors in other cell types to determine if they play a role in regulation of breast epithelial cells and (2) are characterizing a number of putative Cdk inhibitors in breast epithelial cells to elucidate their roles in cell cycle regulation.

(6) BODY

(a) SOW Task1: Characterization of Cdk inhibitor production in normal and malignant breast epithelial cells (months 1-24).

Initial analysis has been focused on members of the p27 inhibitor family, first in normal breast epithelial cells responding to $TGF\beta$. Cells of normal breast strain

184 were synchronized by deprivation of EGF and then released in the presence or absence of TGF β . It had previously been shown that under these circumstances, cyclin E/Cdk2 complexes were inhibited by treatment with TGF β (1). Using an antibody prepared against a peptide of p27 (2) that is highly conserved relative to p21, a structurally related inhibitor, we determined (a) that p27 was not expressed at high levels in normal breast epithelial cells and (b) and that p27 levels did not change significantly in response to TGF β (Fig. 1). On the other hand, several breast carcinoma derived cell lines did express p27 at significant levels (Table 1). We therefore concluded that regulation of p27 levels, or its lack, was not likely to be central to TGF β regulation of breast epithelial cell lines.

The p27 peptide antibodies, however, recognized other proteins in breast epithelial cells of molecular weight 57 kD and 25 kD, respectively. Because the antibodies used were raised against a region highly conserved between p27 and p21, it was thought conceivable that these other proteins might be related inhibitors. We therefore initiated their characterization (discussed under Task 5).

Methods: The methods employed here are standard preparation of protein lysates from mammalian tissue culture cells and Western blotting by the ECL method.

Preparation of the antisera are discussed in Ref. 2.

(b) SOW Task 2: Characterization of cyclin/Cdk complexes in normal and malignant breast epithelial cells (months 1-36).

We have characterized cyclin E/Cdk2 and cyclin A/Cdk2 complexes from normal breast epithelial cells and a number cell lines derived from breast carcinomas for responsiveness to Cdk inhibitors (Fig. 2). Boiled extracts were

prepared from quiescent or TGF β -treated breast epithelial cells as a source of inhibitors that accumulate under these circumstances. We, however, are still not certain concerning which inhibitors are the most prominent in these boiled extracts. These extracts were then used to treat immunoprecipitated cyclin E/Cdk2 or cyclin A/Cdk2 complexes prior to the standard histone H1 kinase assay (3). We found that one cell line, MDA-MB-231, produced a cyclin E/Cdk2 but not a cyclin A/Cdk2 that was reproducibly resistant to inhibition (Fig. 2). This allowed us to infer that the basis for the resistance was likely to be in an alteration of cyclin E structure or function rather than of Cdk2 structure or function.

Methods: the procedures employed involve the preparation of boiled lysates containing inhibitors from mammalian tissue culture cells and their use in immune complex cyclin dependent kinase histone H1 assays, as described in Ref. 1.

We, therefore set out to clone the cyclin E cDNA from MDA-MB-231. PCR primers were designed to amplify the coding region by RT-PCR and poly A RNA was prepared from this cell line, as well as from normal breast epithelial cells. Cyclin E clones obtained in this manner were subject to DNA sequence analysis. All clones obtained from the normal breast epithelial cells conformed to published sequence of cyclin E (data not shown). However several of the clones from MDA-MB-231 contained a three codon deletion at amino acids 23 through 25 (Fig. 3), suggesting that this mutant form might account for the resistance to inhibitors. We are currently preparing recombinant cyclin E/Cdk2 kinase containing this cyclin E deletion mutation in yeast to determine if such complexes are resistant to known Cdk inhibitors in an in vitro assay.

Methods: the procedures employed are standard molecular biology protocols.

(c) SOW Task 3: Test of essentiality of cell cycle regulatory components in breast epithelial cells by antisense (months 1-12).

In the initial proposal, we suggested employing phosphorothioate-substituted antisense oligonucleotides to test the essentiality of various cell cycle components. Initial experiments using such oligonucleotides directed against the cyclin E mRNA were disappointing in that the oligonucleotides were toxic at levels required for observation of antisense effects. Specifically, control oligonucleotides at high concentration caused G1 arrest (data not shown). Since this was a possible phenotype anticipated for ablation of cyclin E mRNA by antisense, this approach was deemed unworkable.

We have therefore embarked on an alternative antisense approach, antisense ribozymes. Ribozymes are catalytic RNAs. The intent is to use RNA complementarity to target known RNA endonucleolytic ribozymes to specific sequences in target mRNAs. The approach poses two problems that need to be addressed experimentally. First, a means needs to be found for choosing accessible cleavage sites on non-denatured target RNA. Second, a vehicle for conditionally expressing the ribozyme at high levels in breast epithelial cells needs to be explored. In order to find accessible sites, we have used a library of random 8-mer sequences flanking the hammerhead ribozyme (4). The library is incubated with non-denatured *in vitro* transcribed cyclin E RNA and then 5' RACE is used to identify the favored cleavage sites. Specifically, after library cleavage, a 3' primer is used to reverse transcribe the RNA. Products are then oligonucleotide-tailed and subjected to PCR. In this manner, cDNAs corresponding to cleavage products are amplified, cloned and subjected to sequence analysis. For cyclin E, we have performed the 5'

race procedure and amplify two cleavage products of 800 and 400 bp starting with a primer immediately 3' to end of the coding region (data not shown). These have been cloned and are undergoing sequence analysis to determine the favored cleavage sites.

Methods: the methods used are essentially those described in Ref. 4, with some modifications, notably those involving adaptation for conditional expression so that stable cell lines can be produced.

In order to perform experiments on breast epithelial cells, a conditional expression system needs to be developed. We have chosen to set up the conditional Tta (tetracycline transactivator) system (5) in a chemically immortalized derivative of the 184 breast epithelial strain, known as 184A (6). Initial attempts to establish the Tta system in 184A failed because the cell line is refractory to transfection (data not shown). However a derivative of 184A that transfects well was obtained so that now 184A-Tta cell lines have been made that in transient assays expresses a beta-galactosidase reporter construct under tetracycline control. This will be used for conditional expression of antisense ribozymes.

In order to insure stability of ribozymes once expressed, a cloning vector has been developed that embeds the antisense ribozyme sequence in the VA1 RNA sequence of adenovirus (4). This RNA is known for its in vivo stability. In order to adapt it to the context of conditional expression using the Tta system, strong polIII promoter elements were deleted from vector sequences. We are therefore now in a position to construct antisense ribozymes based on in vitro cleavage data that can be conditionally but efficiently expressed in breast epithelial cells.

Methods: standard molecular biology procedures were used.

(d) SOW Task 4: Cloning and characterization of Cdk inhibitors from HeLa cells (months 1-36).

We had observed that a Cdk inhibitory activity accumulated in HeLa cells in response to the drug lovastatin. Since HeLa cells can be grown inexpensively and in bulk, we decided to identify and characterize this inhibitor and then determine if it is relevant to cell cycle control in breast epithelial cells. Therefore 50 l of non-adherent HeLa cells were treated with lovastatin (7) and a 28 kD heat-resistant inhibitor was purified (2). Purified protein was subjected to tryptic proteolysis and peptide sequences were obtained (2; Fig. 4). Interestingly, several of these showed relatedness to a previously-described inhibitor, p21, convincing us we had identified another inhibitor. We then used the peptide sequences and degenerate PCR primers to clone a fragment of the corresponding cDNA and this to clone the entire cDNA using phage plaque lifts (data not shown). The coding region of this protein when expressed in *E. coli* produces a strong heat resistant Cdk-inhibitory activity (Fig. 5). Shortly after we obtained these results, two papers describing the sequence of murine p27^{Kip1} were published (8,9). We concluded that we had purified and cloned the human version of the same protein.

Methods: the purification of p27 from HeLa cell lysates and the subsequent cloning of the cDNA are described in Ref. 2.

Antibodies were made against both p27 peptides and the entire recombinant protein. One peptide (NH₂-CRNLFGPVDHEELTRDLE-COOH) was chosen

because it was highly conserved between p27 and p21 (2). This antibody was then used to confirm that p27 was indeed regulated in HeLa cells. In the course of lovastatin treatment, p27 was shown to increase, although, interestingly, the corresponding mRNA did not (2; Fig. 6). Likewise, p27 was shown to undergo strong cell cycle variation, with maximal accumulation in G1, although again the p27 mRNA did not fluctuate (2; Fig. 7). These observations led us to conclude that p27 accumulation was regulated at the post-transcriptional level, either by translational control or control of protein stability. Pulse-chase experiments indicated that the regulation is translational (2; Fig. 8). p27 appears to be strongly regulated in a number of other human cell systems as well including fibroblasts and HL60 promyelocytic leukemia cells (2).

Methods: procedures used in the analysis of p27, as described above, are in Ref. 2.

However, as stated above, when the same antibodies were applied to normal breast epithelial cells, low levels of p27 were detected (Fig. 1) compared to these other systems. As a result, we feel it is unlikely that p27 plays a major regulatory role in these cells. It is interesting to note that p27 levels are much higher in an immortalized breast epithelial derivative, 184A (data not shown), indicating that relatively modest genetic alterations can change the internal regulatory environment of the cell significantly.

(e) SOW Task 5: Cloning and characterization of breast epithelial cell Cdk inhibitors (months 12-48).

Since p27, cloned based on our work in HeLa cells, did not appear to be relevant to regulation of normal breast epithelial cells, we decided to shift our focus to other potential inhibitors in breast epithelial cells. As is clear from Fig. 1, at least two other species appear to be strongly cross-reactive with an antibody generated against a conserved peptide of p27: one at 25 kD and one at 57 kD. In order to clone these, we prepared degenerate oligonucleotides based on core regions of homology between p27 and p21. This strategy depends on the assumption that p25 and p57 will be sufficiently related to p27 and p21 to maintain these conserved motifs. In support of this relatedness, p25 and to some extent p57 are heat stable, an unusual characteristic of p21 and p27 (data not shown). Using the degenerate oligo strategy, a fragment closely related to p27 was cloned from human breast cDNA (Fig. 9). Subsequent to this, the sequence of human p57^{Kip2} was published by others (10) and we determined that it was identical to the sequence that we had cloned. However, p57 does not vary in response to TGF β treatment or growth factor deprivation in human breast epithelial cells. Therefore its significance to cell cycle regulation in this system remains unclear. We are attempting to identify conditions during which p57 levels are regulated in normal breast epithelial cells.

Methods: standard molecular biology procedures were employed.

So far attempts to clone p25 have been unsuccessful. We therefore conclude that our primers are inappropriate for amplifying it. Therefore, since we can also detect p25 in HeLa cells, we are initiating a purification of the protein so that the cDNA can be cloned via peptide sequences, as was performed for p27.

(f) SOW Task 6: Characterization of Cdk inhibitors in vivo (months 24-48). We have not yet initiated this phase of the project.

(7) CONCLUSIONS

Our hypothesis that aspects of the cell cycle machinery may be mutated in breast cancer appears to be confirmed. One out of five breast carcinoma-derived cell lines tested, one produced a cyclin E/Cdk2 kinase that was resistant to inhibition by inhibitors found in extracts from normal breast epithelial cells. When cyclin E cDNA was cloned from this cell line a three-codon deletion was found in the coding region. It, however, remains to be determined if this mutation confers resistance to inhibitors.

On the other hand our hypothesis that we could purify and clone an inhibitor from HeLa cells and that this would be the same inhibitor that accumulates in EGF-starved or TGF β -treated breast epithelial cells was not confirmed. The inhibitor isolated from HeLa cells, p27Kip1, was found to be regulated and important in a number of other cell systems but was expressed at low levels in normal breast epithelial cells and levels did not change in response to treatment with TGF β . However, we cannot rule out a role for p27 under these circumstances. It has been recently been shown that in mink lung epithelial and human keratinocytes that p27, although its steady state level doesn't change in response to treatment with TGF β , may be displaced onto cyclin E/Cdk2 complexes from cyclin D/Cdk4 complexes (11). Thus, some inhibition of cyclin E/Cdk2 may be occurring in breast epithelial cells by a similar mechanism.

Finally, in the course of analyzing p27 in breast epithelial cells, we produced evidence for at least two other related molecules that are expressed at higher levels in breast epithelial cells. p57, although expressed at higher levels than p27, also does not appear to be regulated directly by TGF β . However, we cannot rule out an indirect displacement model as has been described for p27 in mink lung epithelial cells. We are currently pursuing this idea. In addition, we are screening for other antiproliferative signals that might affect p57 levels or function in breast epithelial cells, such as treatment with interferons. p25, on the other hand, has been difficult to analyze because so far we have been unable to clone the corresponding cDNA. This goal is still being pursued.

The initial plan to use chemically substituted antisense oligonucleotides to test the essentiality of gene products proved unworkable. This has been a consistent finding in the antisense field. Except in unusual cases, the toxicity associated with these modified oligonucleotides precludes their being used at high enough concentrations to produce antisense effects. We have therefore embarked on an alternative but significantly more complicated strategy employing antisense ribozymes. This has involved finding target sites, developing a suitable expression vector and finding a breast epithelial cell line that would transfect efficiently. These have essentially been accomplished. It should be possible now to return to the original experimental issues involving the essentiality of various cyclins and Cdks.

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Table 1. p27 status of breast carcinoma-derived cell lines.

Cell Line	p27 status *
184 (WT)	+/-
MDA-MB-231	+/-
T47D	++
MDA-MB-157	-
ZR-75-30	++

* Relative p27 status was determined by harvesting cultures of asynchronous cells and preparing lysates of total cellular protein followed by Western blotting with a p27-specific antibody. +/-, the level detected in a normal strain of breast epithelial cells (184) indicates a marginal but detectable level. However, we don't think this level is sufficient to have serious implications for cell cycle control.

Figure Legends:

Figure 1. p27 related proteins in normal breast epithelial cells emerging from quiescence and treated with TGF β . Asynchronous normal breast epithelial cells (strain 184) (lane 1) were rendered quiescent by EGF deprivation as described in Ref. 6 (lane 2) and then stimulated to re-enter the cell cycle for 8 hours (lane 3), 16 hours (lane 4) and 24 hours (lane 5). A parallel sample was treated with TGF β , as described (6), for 16 hours. Total cellular lysates were prepared, subjected to SDS-PAGE, and Western blotted using antibodies prepared against a peptide of p27 highly conserved in p21 and almost completely conserved in p57. Reactive species at 25 and 57 kD are indicated. Position of human p27 is indicated although not visible on this blot.

Figure 2. MDA-MB-231 contains a cyclin E/CDK2 kinase that is resistant to Cdk inhibitors from breast epithelial cell extracts. Cyclin E/CDK2 and cyclin A/CDK2 kinase complexes were prepared from breast carcinoma cell line MDA-MB-231 by immunoprecipitation. Histone H1 kinase assays were performed after incubation with either buffer (cont) or boiled extracts from either EGF-starved (Go) or TGF β -treated (TGF) normal breast epithelial cells. Two different inhibitory extracts of each type were assayed. The cyclin E/CDK2 complexes from this cell line were resistant to inhibition whereas the cyclin A/CDK2 complexes were not. Cyclin E/CDK2 complexes from normal breast epithelial cells and four other breast carcinoma-derived cell lines were inhibitable by these extracts (data not shown).

Figure 3. Breast carcinoma cell line MDA-MB-231 expresses a cyclin E mRNA containing a three-codon deletion. Cyclin E-specific cDNA was prepared from

breast carcinoma cell line MDA-MB-231 and subjected to sequence analysis.

Several cDNAs were found to contain a three-codon deletion encompassing codons 23-25.

Figure 4. Sequences of tryptic peptides obtained from purified 28 kD inhibitor from lovastatin arrested HeLa cells. All sequences were found eventually to be contained in human p27.

Figure 5. Inhibitory activity of recombinant 28 kD inhibitor on cyclin A/CDK2 kinase activity. The open reading frame corresponding to the putative 28 kD inhibitor was cloned into *E. coli* expression vector pET3c. The soluble fraction of heat treated extracts from cells transformed with this plasmid contained a single species of 28 kD. Increasing amounts of this extract were added to a fixed amount of cyclin A/CDK2 immunoprecipitated on protein A sepharase beads prior to performing a histone H1 kinase assay. Activity is given as the percentage of input kinase activity.

Figure 6. Post-transcriptional regulation of p27 induction in lovastatin arrested cells. HeLa cells were treated with lovastatin, and samples were taken over a 60 hour time course. The distribution of cell cells in G1, S., or G2/M phases was determined by FACS analysis of BrDU pulse-labelled cells. Protein extracts from each time point were analyzed for p27 and cyclin E protein levels. RNA was isolated from the same samples and hybridized with p27 specific probe and , as a control, with a glyceraldehyde phosphate dehydrogenase (GAPDH) specific probe.

Figure 7. Post-transcriptional regulation of p27 through the cell cycle. HeLa cells were synchronized using a thymidine/nocodazole block/release protocol. Time

points were taken every hour after release from the nocodazole block. The degree of cell cycle synchrony was determined by FACS analysis. Heast stable Cdk inhibitory activity was determined using cyclin A associated kinase as a substrate. Protein extracts from each time point were analyzed for p27 levels, cyclins E and A, or CDKS. RNA was isolated from the same samples and hybridized with a p27 specific probe and, as a control, with a glyceraldehyde phosphate dehydrogenase specific probe.

Figure 8. Translational control of p27 levels in HeLa cells. A. HeLa cells were pulse-labelled for 1 hour with a mixture of ^{35}S -methionine and ^{35}S -cysteine and incubated in the presence of an excess of non-radioactive methionine and cystein for additional times as indicated (chase). Cells were lysed and p27 was immunoprecipitated. Aliquots of each sample used in the immunoprecipitation were separated by SDS-PAGE and are shown as Coomassie Brilliant Blue proteins (B).

Figure 9. Nucleotide and deduced protein sequence obtained by PCR using p21/p27 consensus degenerate primers. The fragment obtained is shown to correspond to human p57 (10).

Figure 1

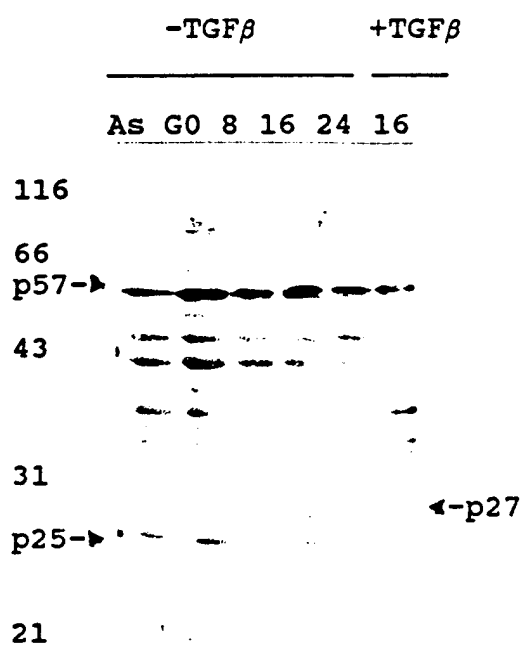


Figure 2

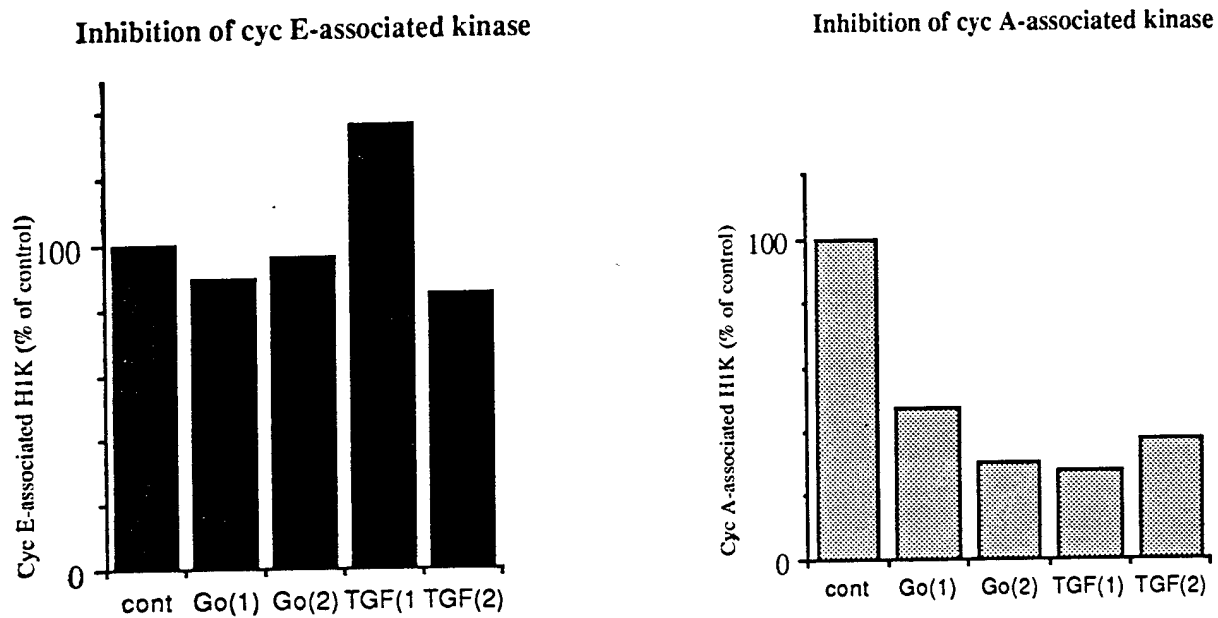


Figure 3

52	gca aac gtg acc gtt	ttt ttg cag	gat cca gat gaa gaa	WILD TYPE
18	ala asn val thr val	phe leu gln	asp pro asp glu glu	

deletion

52	gca aac gtg acc gtt	gat cca gat gaa gaa	231 ISOLATE
18	ala asn val thr val	asp pro asp glu glu	

Sequence comparison of wild type cyclin E versus the one of 231 strain

Figure 4

Tryptic Peptides Derived from purified 28 kD CDK inhibitor.

- (1) $\text{NH}_2\text{-NLFGPDHEEL-COOH}$
- (2) $\text{NH}_2\text{-NDFQNHKP-COOH}$
- (3) $\text{NH}_2\text{-YEWQEVEK-COOH}$
- (4) $\text{NH}_2\text{-LPEFYYP-COOH}$
- (5) $\text{NH}_2\text{-RPQFR-COOH}$

Figure 5

Inhibition of cyclin A / Cdk2 by recombinant p27

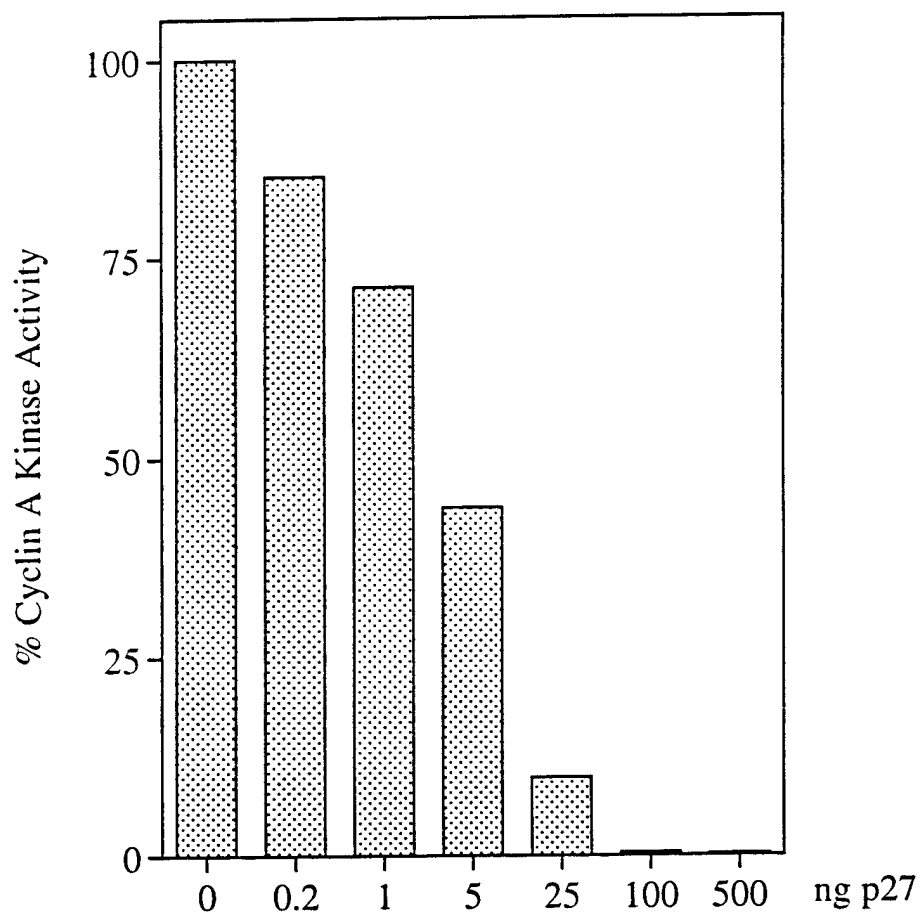
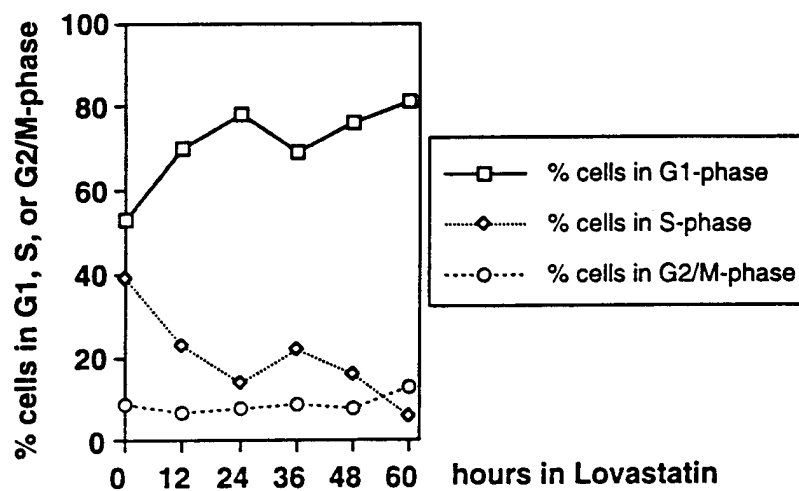


Figure 6



 α -p27

 α -Cyclin E

 p27 mRNA

 GAP-DH mRNA

Figure 7

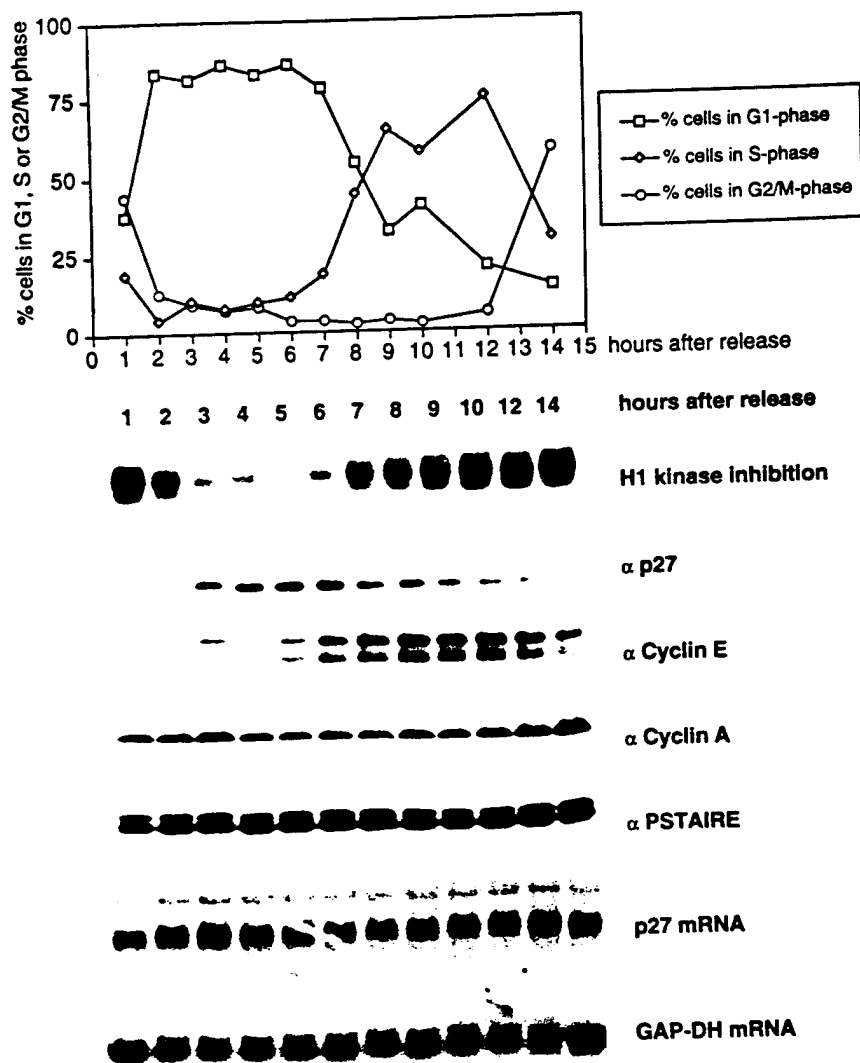


Figure 8

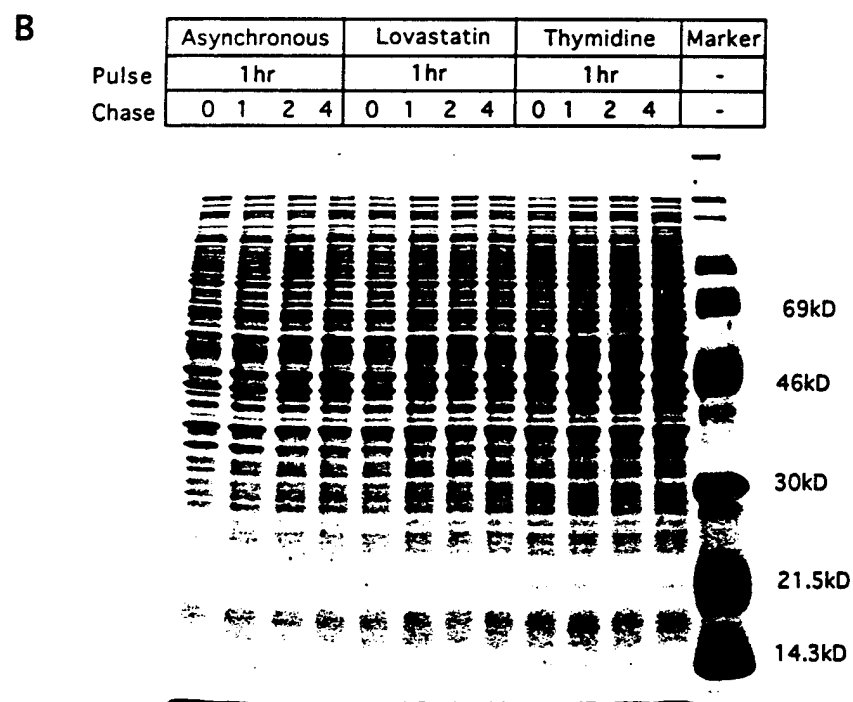
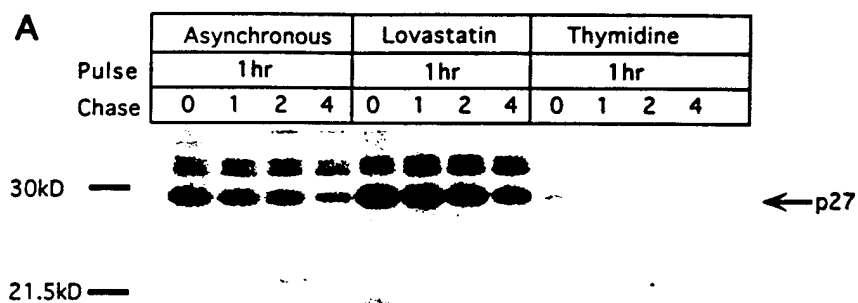


Figure 9

gag ctg agc cgc gag ctg cag gcc cgc ctg gcc gag ctg aac gcc gag gac cag aac cgc
glu leu ser arg glu leu gln ala arg leu ala glu leu asn ala glu asp gln asn arg

Nucleotide and deduced protein sequence of the clone PCR1

PCR1										E	L	S	R	E	L	Q	A	R	L	A	E	L	N	A	E	D	Q	N	R			
p57	G	P	V	D	H	E	E	L	S	R	E	L	Q	A	R	L	A	E	L	N	A	E	D	Q	N	R	W	D	Y	D	F	Q
	*	*	*	*	*	*																				*	*	*	*	*	*	
p27	G	P	V	D	H	E	E	L	T	R	D	L	E	K	H	C	R	D	M	E	E	A	S	Q	R	K	W	N	F	D	F	Q

Sequence comparison of PCR1 with p57^{kip2} and p27^{kip1}

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